Corn Agmatine Iminohydrolase

PURIFICATION AND PROPERTIES

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ABSTRACT

Agmatine iminohydrolase (EC 3.5.3.12) was purified 7,300-fold from extracts of corn shoots by chromatographic separations on diethylaminoethyl-cellulose, Sephadex G-100, and agmatine-affinity column. The enzyme was homogeneous by the criteria of analytical gel electrophoresis. Molecular weight estimated by Bio-Gel P-200 was 85,000, and the enzyme seems to be a dimer with identical subunits (molecular weight, 43,000). The isoelectric point determined by gel electrophoresis was 4.7. The optimal pH and temperature for activity were 6.5 and 60°C, respectively. The activation energy was 10.9 kilocalories per mole. High specificity exists for agmatine, the K\text{m} value for agmatine was 1.9 × 10^{-4} mol, and the enzyme was present in the cytosol. The enzyme was sensitive to Cu^{2+} and Zn^{2+} and also was inhibited by \( p \)-hydroxymercuribenzoate and aracain.

Putrescine in some higher plants seems to be formed via NCP\textsuperscript{2} (15, 20). The discovery of agmatine iminohydrolase in corn, sunflower (14), and groundnut (13) provided \textit{a priori} evidence for enzymatic formation of NCP from agmatine. Agmatine iminohydrolase catalyzes the hydrolysis of agmatine to NCP and ammonia as follows:

\[
\text{agmatine} + \text{H}_2\text{O} \rightarrow \text{NCP} + \text{NH}_3
\]

Although the enzyme has been purified (about 375-fold) from groundnut cotyledons (13), no attempts seem to have been made so far to obtain the enzyme in homogeneous form from corn or any other plant source. We report here a procedure for the purification of agmatine iminohydrolase from corn shoots and describe the enzyme properties.

MATERIALS AND METHODS

Plant Material. Seeds of corn (Zea mays L. cv. Goldencross Bantam T51) were germinated in moist vermiculite at 25°C for 2 days in the dark. Seedlings were transferred to plastic trays containing Hoagland solution. They were grown under continuous light (about 3,000 lux at plant level) at 25°C for 10 to 14 days. The harvested shoots were washed thoroughly with 0.1% benzalkonium chloride solution, rinsed with deionized \( \text{H}_2\text{O} \), and used for the experiment.

Chemicals. Caprylohydroxamic acid was a gift of Dr. Kobashi. NCP (m.p., 185°C) was prepared by the method of Smith and Carraway (15) and was recrystallized from ethanol. Agmatine sulfate, arcan sulfide, reduced glutathione, \( p \)-HMB, \( N \)-ethylmaleimide, iodoacetamide, PVPP, catalase, BSA, ovalbumin, chymotrypsinogen, and Cyt c were purchased from Sigma. Sephadex G-100, Sephadex G-25, and Sepharose 6B were obtained from the Pharmacia Fine Chemicals. Bio-Gel P-200 was obtained from Bio-Rad Laboratories. DEAE-cellulose was purchased from Brown Co. All other chemicals used were of analytical reagent grade. Agmatine-SA-AH-Sepharose 6B was prepared according to the method of Cuatrecasas and Anfinsen (5).

Enzyme Assay. Enzyme activity was routinely assayed by measuring the ammonia released from agmatine in Conway microdiffusion units. The liberated ammonia was determined with Nessler's reagent. The standard assay mixture, consisting of 200 \( \mu \text{mol} \) phosphate buffer (\( \text{pH} 6.5 \)), 30 \( \mu \text{mol} \) agmatine sulfate, 1 \( \mu \text{mol} \) chloramphenicol, 1 \( \mu \text{mol} \) streptomycin sulfate, and enzyme solution in a total volume of 2.0 ml, was incubated in a test tube for 2 h at 30°C. The reaction was initiated by adding the enzyme and terminated by the addition of 0.1 ml 50% trichloroacetic acid.

One unit is defined as the amount of enzyme liberating 1 \( \mu \text{mol} \) ammonia in 1 h. Specific activity is presented as units/mg protein.

Protein Determination. Protein was determined by the Lowry procedure (9), with BSA as the standard.

Subcellular Distribution Study. For determining the subcellular distribution of enzyme activity, shoots (10 g) were blended in a mortar with 2 volumes (v/w) of 50 mm Tris-HCl buffer (\( \text{pH} 7.0 \)) containing 0.45 M sucrose and PVPP (0.1 g/g fresh weight). The macerate was subjected to differential centrifugation as indicated in Table II.

Stoichiometry Studies. The reaction mixture per 2.0 ml contained the following compounds: 10 \( \mu \text{mol} \) agmatine sulfate, 2.3 \( \mu \text{mol} \) \( p \)-hydroxymercuribenzoate, 2.3 \( \mu \text{mol} \) \( p \)-hydroxymercuribenzoate, and 200 \( \mu \text{mol} \) phosphate buffer (\( \text{pH} 6.5 \)). Incubation was carried out in test tubes for 1 h at 30°C. The remaining agmatine and NCP were separated by high-voltage paper electrophoresis (3,000 \( \text{v}, 15 \text{ min} \) in 1 M acetic acid) and estimated colorimetrically (7, 16). Ammonia was estimated by the microdiffusion method with Nessler's reagent (3, 17).

Analytical Polyacrylamide Gel Electrophoresis. Disc gel electrophoresis was performed at two different pH values, 9.4 and 8.0, was described by Davis (6). Electrophoresis on 7.5% acrylamide gels (0.5 × 6.6 cm) was run at 3 mamp/gel column and gels were stained 0.1% Coomassie brilliant blue in 7% acetic acid for protein and then destained in 7% acetic acid solution.

Determination of Molecular Weight. The molecular weight of the enzyme was estimated on a Bio-Gel P-200 column according to the method of Andrews (2). The column was calibrated with catalase (mol wt, 232,000), BSA dimer (mol wt, 136,000), and ovalbumin (mol wt, 45,000), as markers of known molecular weight.

SDS Electrophoresis. SDS electrophoresis was carried out according to Weber and Osborn (18). Purified enzyme (3 \( \mu \text{g} \) from

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2 Abbreviations: NCP, \( N \)-carbamylputrescine; \( p \)-HMB, sodium \( p \)-hydroxymercuribenzoate; PVPP, polyvinylpolypyrrolidone; Agmatine-SA-AH-Sepharose 6B, agmatine-succinic acid-diaminoxhexane-Sepharose 6B.
RESULTS

PURIFICATION OF ENZYME

Step 1: Crude Extract. The shoots (450 g) were blended in a chilled Waring blender with PVPP (0.1 g/g fresh weight) and 2 volumes (v/w) of the extracting buffer [50 mm phosphate buffer (pH 6.5) containing 5 mm 2-mercaptoethanol and 0.1 mm EDTA]. The homogenate was filtered through cheesecloth and clarified by centrifugation (10,000g, 15 min).

Step 2: Adsorption and Elution on DEAE-Cellulose. The supernatant of Step 1 was stirred with DEAE-cellulose equilibrated with the extracting buffer described above (supernatant/DEAE-

Step 5, see below) was treated with 1% SDS and 2-mercaptoethanol for 10 min at 100 C and then subjected to SDS electrophoresis on 5% gels to determine the subunit nature of the enzyme.

Isoelectric Focusing. The isoelectric point of the enzyme was determined by the gel-electrofocusing method (19). The pH values were measured with a Hitachi-Horiba M-5 pH meter.

Table I. Purification of Corn Agmatine Iminohydrolase

<table>
<thead>
<tr>
<th>Step</th>
<th>Total Protein (mg)</th>
<th>Total Activity (units)</th>
<th>Specific Activity (units/mg)</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Crude extract</td>
<td>5,992.5</td>
<td>719.1</td>
<td>0.12</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>2. DEAE-cellulose eluate</td>
<td>684.5</td>
<td>573</td>
<td>0.84</td>
<td>7</td>
<td>79.7</td>
</tr>
<tr>
<td>3. Eluate from DEAE-cellulose column</td>
<td>142.8</td>
<td>429.8</td>
<td>3.01</td>
<td>25.1</td>
<td>59.8</td>
</tr>
<tr>
<td>4. Sephadex G-100</td>
<td>28.2</td>
<td>409.8</td>
<td>14.52</td>
<td>121</td>
<td>57.0</td>
</tr>
<tr>
<td>5. Eluate from affinity column</td>
<td>0.19</td>
<td>166.5</td>
<td>876.31</td>
<td>7,302.5</td>
<td>23.2</td>
</tr>
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</table>

Table II. Subcellular Distribution of Corn Agmatine Iminohydrolase

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Specific Activity (units/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant, 5 × 10^3 g</td>
<td>0.06</td>
</tr>
<tr>
<td>Pellet, 5 x 10^2 to 2 x 10^2 g</td>
<td>0</td>
</tr>
<tr>
<td>Pellet, 2 x 10^2 to 10^2 g</td>
<td>0</td>
</tr>
<tr>
<td>Pellet, 10^2 to 10^3 g</td>
<td>0</td>
</tr>
<tr>
<td>Supernatant, 10^3 g</td>
<td>0.14</td>
</tr>
</tbody>
</table>

Step 3: Column Chromatography on DEAE-Cellulose. The concentrated enzyme fraction of Step 2 was applied to a column (2.2 × 35 cm) of DEAE-cellulose pre-equilibrated with the extracting buffer. A pH gradient was established with 400 ml of the extracting buffer in the mixing flask and 400 ml 0.5 M KH2PO4 solution containing 5 mm 2-mercaptoethanol and 0.1 mm EDTA in the reservoir. The active fractions (pH 6.2 to 5.7) were reduced in volume by a concentrator (Toyomodel UHP 43).

Step 4: Sephadex G-100 Filtration. The concentrated fraction of Step 3 was subjected to Sephadex G-100 filtration. After the
Sephadex G-100 column (2.2 × 70 cm) was equilibrated with the extracting buffer, the enzyme solution (Step 3) was loaded on the column and the buffer was allowed to flow at a rate of 10 ml/h. Active fractions were pooled and dialyzed against 50 mM phosphate buffer (pH 8.5) containing 5 mM 2-mercaptoethanol and 0.1 mM EDTA.

**Step 5: Agmatine-SA-AH-Sepharose 6B Affinity Chromatography.** The dialyzed enzyme of Step 4 (containing approximately 15 mg protein) was applied to a column (1.0 × 5.0 cm) equilibrated with 50 mM phosphate buffer (pH 8.5) containing 5 mM 2-mercaptoethanol and 0.1 mM EDTA. After 60 ml 0.5 mM phosphate buffer (pH 8.5) containing 5 mM 2-mercaptoethanol and 0.1 mM EDTA was passed through the column, the bound enzyme was eluted with 10 ml of the same buffer containing 10 mM agmatine sulfate (Fig. 1, arrows). The flow rate was approximately 25 ml/h. Active fractions were pooled and dialyzed against the extracting buffer. A summary of the purification procedure is given in Table I.

**SUBCELLULAR DISTRIBUTION OF ENZYME**

The subcellular localization of agmatine iminohydrolase is strongly indicated in Table II. Enzyme activity was found only in the cytosol fraction. This agrees with Smith’s suggestion (14).

**CRITERIA OF PURITY**

Polyacrylamide-disc gel electrophoresis of the purified enzyme showed a single band at both pH 9.4 and 8.0 (Fig. 2).

**EFFECT OF pH AND TEMPERATURE**

The enzyme exhibited a single pH optimum at 6.5, activity rapidly declining below pH 6.0 and declining less rapidly above pH 7.0 (Fig. 3). The purified enzyme (concentration, 8 μg/ml) lost 30% of the activity in 3 days at 4°C. Exposure of the enzyme to 50°C for 10 min resulted in a decrease of approximately 50% in the activity. Optimum temperature was 60°C and energy of activation between 30 and 50°C was 10.9 kcal/mol.

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**Table III. Stoichiometry for Conversion of Agmatine to N-Carbamylputrescine and Ammonia**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Agmatine</th>
<th>N-Carbamylputrescine</th>
<th>NH₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.9</td>
<td>2.4</td>
<td>2.0</td>
</tr>
<tr>
<td>2</td>
<td>4.2</td>
<td>4.2</td>
<td>3.8</td>
</tr>
</tbody>
</table>

**Table IV. Protection and Reactivation by GSH of Agmatine Iminohydrolase Activity against Inhibition by p-HMB**

<table>
<thead>
<tr>
<th>Order of Addition</th>
<th>AG</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>A → B → C</td>
<td>AG</td>
<td>0</td>
</tr>
<tr>
<td>GSH</td>
<td>AG</td>
<td>0</td>
</tr>
<tr>
<td>p-HMB</td>
<td>AG</td>
<td>33.7</td>
</tr>
<tr>
<td>AG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-HMB</td>
<td>AG</td>
<td>93.7</td>
</tr>
<tr>
<td>GSH</td>
<td>AG</td>
<td>3.1</td>
</tr>
<tr>
<td>p-HMB</td>
<td>GSH</td>
<td>45.0</td>
</tr>
</tbody>
</table>

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**Fig. 5.** SDS-gel electrophoresis of purified agmatine iminohydrolase. A, untreated enzyme; B, enzyme treated with 1% SDS and 2-mercaptoethanol at 100°C for 10 min.

**Fig. 6.** Determination of molecular weight by SDS electrophoresis. A, BSA; B, ovalbumin; C, chymotrypsinogen; D, Cyt c; E, agmatine iminohydrolase.

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STOICHIOMETRY

The stochiometry of the agmatine iminohydrolase reaction was obtained by measuring the disappearance of agmatine and the appearance of NCP and ammonia. Approximately 1 mol each of NCP and ammonia were produced from 1 mol agmatine by the enzyme fraction of Step 5 of the purification procedure (Table III).

MOLECULAR WEIGHT AND SUBUNITs

A molecular weight of approximately 85,000 was obtained with the Bio-Gel P-200 gel filtration (Fig. 4) technique. SDS-disc gel electrophoresis showed a single band with a mol wt of 43,000 (Figs. 5 and 6). From these data, it is suggested that native corn agmatine iminohydrolase is composed of two identical subunits.

ISELECTRIC POINT

The isoelectric point of corn agmatine iminohydrolase was determined to be 4.7 by gel electrofocusing.

SUBSTRATE SPECIFICITY

Under standard assay conditions, corn agmatine iminohydrolase was found to be substrate-specific. No detectable activity was found with arginine, creatine, creatinine, and glycocoyamine (each 15 mM) as substrates.

EFFECT OF AGMATINE CONCENTRATION

Under standard assay conditions, activity was linear with time and proportional to protein content. The enzyme showed a hyperbolic response to increasing agmatine concentrations. A Line-weaver-Burk plot gave a $K_{m}$ of 1.9 $\times$ 10^{-4} M.

EFFECT OF METAL IONS AND SOME INHIBITORS

The enzyme was inhibited by several divalent cations, such as Cu^{2+}, Zn^{2+}, Co^{2+}, Fe^{2+}, Ni^{2+}, Ca^{2+}, and Mg^{2+} (each 1 mM). The addition of Cu^{2+}, Zn^{2+}, and Co^{2+} resulted in inhibitions of 100, 85, and 55%, respectively. EDTA (10 mM), o-phenanthroline, diethylthiocarbamate, semicarbazide, and NaF (each 1 mM) had no effect. Carpylydroxamic acid and acetohydroxamic acid (each 0.1 mM), frequently used as inhibitors of urease (8), were without effect. Aracain has been reported as an effective inhibitor (14). Our purified enzyme was inhibited by it (about 28% at 1 mM and 41% at 2 mM). On the other hand, spermine, spermidine, putrescine, arginine, ornithine, citrulline, and urea (each 2 mM) had no effect.

EFFECT OF SULFHYDRL REAGENTS

The enzyme was inhibited by p-HMB (Table IV). When preincubated with p-HMB, the inhibition was extensive; however, when preincubated with reduced glutathione before adding p-HMB, it was protected. The p-HMB-inhibited enzyme was reactivated partially by adding reduced glutathione (Table IV). N-Ethylmal- eimide and iodoacetamide (each 1 mM) were not inhibitory.

DISCUSSION

Agmatine iminohydrolase was first detected in corn seedlings in 1969 (14) but, until now, has not been purified. Recently, the enzyme from groundnut cotyledons was purified about 375-fold, but the criteria of purity were not delineated (13). The purification obtained by the method described herein was 7,300-fold, and the protein was electrophoretically homogeneous. With the Bio-Gel P-200 gel filtration method, the mol wt of the enzyme was found to be approximately 85,000, whereas SDS-gel electrophoresis technique gave a mol wt of 43,000. It is, therefore, suggested that the enzyme is a dimer. Smith (14) obtained a value of 19.0 kcal/mol for the activation energy of this enzyme. Here, this value was 10.9 kcal/mol, and the $K_{m}$ value for agmatine was 1.9 $\times$ 10^{-4} M. Corresponding values for the groundnut enzyme were 22 kcal/mol and 7.6 $\times$ 10^{-4} M, respectively (13). Groundnut enzyme was inhibited by tryptamine, putrescine, cadaverine, spermine, and spermidine (13); however, these compounds had no effect on corn enzyme. A possible reason for this discrepancy may reside in the differences in the assay procedures. In microorganisms, agmatine is degraded by two different enzyme systems, namely agmatine iminohydrolase (12) and agmatinase (EC 3.5.3.11) (11). However, our stoichiometric studies indicated that the purified enzyme from corn shoots catalyzed only the hydrolysis of agmatine to NCP and ammonia (Table III). In addition, the presence of urea did not affect ammonia production by the reaction mixture. It is concluded that an agmatinase-urease system was not in our preparation. NCP may be formed directly by decarboxylation of citrulline in sugarcane (10), Sesamum (4), and Escherichia coli (1). However, the properties of citrulline decarboxylase have not been characterized.

Acknowledgment—We wish to thank Prof. K. Kobashi of Toyama Medical and Pharmaceutical University for a generous gift of caprylydroxamic acid.

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